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6. AUTHOR(S) DR GERALD A. LEBLANC		
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Dept of Toxicology North Carolina State University Box 7633 Raleigh NC 27695-7633		8. PERFORMING ORGANIZATION REPORT NUMBER
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13. ABSTRACT (Maximum 200 words)

Biomarkers of chemical exposure and effect have substantial utility in chemical hazard and risk assessments. The overall goal of this research project was to test the hypothesis that changes in the metabolic elimination of the steroid hormone testosterone could serve as a non-invasive biomarker of toxicant exposure and effect in a variety of sentinel species and humans. Objective 1 of the program was to characterize testosterone biotransformation processes in an invertebrate (*Daphnia magna*), a fish (fathead minnow, *Pimephales promelas*), and a mammal (mouse, CD-1 strain). Results from this objective conclusively demonstrated that testosterone is extensively biotransformed by qualitatively similar metabolic pathways in various organisms and that the metabolic elimination of testosterone can be measured using non-invasive approaches. Objective 2 was to evaluate the utility of changes in the metabolic elimination of testosterone as a noninvasive biomarker of toxicant exposure. Results confirmed that various components of the pathways leading to the metabolic elimination of testosterone are susceptible to perturbation resulting from chemical exposure. These perturbations can be readily measured and exploited as noninvasive biomarkers of toxicant exposure. Objective 3 was to establish relationships

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between chemical effects on the metabolic elimination of testosterone (the biomarker) and toxicity to steroid hormone-dependent processes such as reproduction and development (the effect). Results provided considerable evidence that chemical-induced effects on testosterone biotransformation/elimination processes can alter steroid hormone homeostasis resulting in perturbations in certain physiological processes. Taken together, results from this program have demonstrated that changes in the metabolic elimination of testosterone can serve as a sensitive, predictive, and noninvasive biomarker of toxicity associated with hormonal perturbation. This approach holds promise for widespread application in the hazard/risk assessment process.

Final report of grant #: F49620-94-1-0266

**STEROID HYDROXYLASE ACTIVITIES AS NONINVASIVE BIOMARKERS
OF TOXICANT EXPOSURE AND EFFECT**

Gerald A. LeBlanc, Ph.D.
Department of Toxicology
Box 7633
North Carolina State University
Raleigh NC 27695-7633

Prepared for: United States Air Force
Air Force Office of Scientific Research
Building 410
Bolling AFB, D.C. 20332-6448

SUMMARY

Biomarkers of chemical exposure and effect have substantial utility in chemical hazard and risk assessments. The overall goal of this research project was to test the hypothesis that changes in the metabolic elimination of the steroid hormone testosterone could serve as a non-invasive biomarker of toxicant exposure and effect in a variety of sentinel species and humans. Objective 1 of the program was to characterize testosterone biotransformation processes in an invertebrate (*Daphnia magna*), a fish (fathead minnow, *Pimephales promelas*), and a mammal (mouse, CD-1 strain). Results from this objective conclusively demonstrated that testosterone is extensively biotransformed by qualitatively similar metabolic pathways in various organisms and that the metabolic elimination of testosterone can be measured using non-invasive approaches. Objective 2 was to evaluate the utility of changes in the metabolic elimination of testosterone as a noninvasive biomarker of toxicant exposure. Results confirmed that various components of the pathways leading to the metabolic elimination of testosterone are susceptible to perturbation resulting from chemical exposure. These perturbations can be readily measured and exploited as noninvasive biomarkers of toxicant exposure. Objective 3 was to establish relationships between chemical effects on the metabolic elimination of testosterone (the biomarker) and toxicity to steroid hormone-dependent processes such as reproduction and development (the effect). Results provided considerable evidence that chemical-induced effects on testosterone biotransformation/elimination processes can alter steroid hormone homeostasis resulting in perturbations in certain physiological processes. Taken together, results from this program have demonstrated that changes in the metabolic elimination of testosterone can serve as a sensitive, predictive, and noninvasive biomarker of toxicity associated with hormonal perturbation. This approach holds promise for widespread application in the hazard/risk assessment process.

INTRODUCTION

Biomarkers have been extensively used in medicine for the detection and diagnosis of disease. Similarly, biomarkers of toxicant exposure in sentinel species has demonstrated utility in chemical hazard and risk assessments (1). An additional, though under utilized, role of biomarkers is in the prediction of toxicity associated with chemical exposure, that is: *biomarkers as indicators of toxicant effect* (e.g. (2)). The use of predictive biomarkers of toxicant effect would serve to detect chemical exposure before such incidents present themselves as disease or dysfunction. Appropriate intervention measures in response to early biomarkers would thus greatly mitigate health problems associated with chemicals in the environment and workplace.

The overall goal of this project was to test the hypothesis that changes in the metabolic elimination of the steroid hormone testosterone could serve as a non-invasive biomarker of toxicant exposure and effect in a variety of sentinel species. Results, it was anticipated, would provide methodologies for assessing toxicant exposure and effect in feral species (i.e. wildlife inhabiting contaminated areas), implanted sentinels (i.e. caged fish placed in streams suspected of receiving toxicant runoff), and would provide preliminary data on the likelihood that similar methodologies could be developed for the noninvasive evaluation of toxicant exposure and effect in humans (i.e. diagnostic assays).

The individual objectives of the program were greatly exceeded. The initial objective was to characterize testosterone hydroxylase activities in an invertebrate (water flea, *Daphnia magna*), a fish (fathead minnow, *Pimephales promelas*), and a mammal (mouse, CD-1 strain) and exploit these activities as biomarkers of perturbation in

steroid metabolism. In addition to accomplishing this objective, we also characterized steroid conjugative processes (both carbohydrate and sulfate conjugation) as well as a variety of oxido-reductive pathways. The integration of all of these processes in assessing the metabolic elimination of testosterone has greatly increased the sensitivity of the assay as a predictive biomarker of toxicant effect (Aim 3). We also exceeded the initial scope of the proposed program by assessing the effects of xenobiotic exposure on testosterone synthesis and homeostasis and establishing causality between toxicant-induced changes in steroid metabolism and toxicity. A summary of the accomplishments, as related to each project objective, is provided below.

ACCOMPLISHMENTS

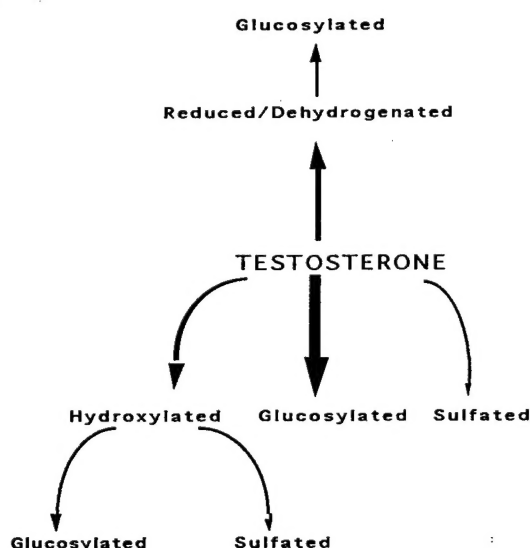
Objective 1 Characterize testosterone biotransformation processes in an invertebrate (*Daphnia magna*), a fish (fathead minnow), and a mammal (mouse). Current concern over the use of vertebrates in experimentation provided the rationale for including the invertebrate model in these studies. Furthermore, daphnids are extensively used in toxicity testing of environmental chemicals (e.g. (3)) and methodologies generated in this study could be incorporated into standard toxicity assessments to provide mechanistic information regarding the toxicity of some chemicals. Fish were selected for their potential use as sentinel species of environmental contamination, and mouse was used as a model to assess the application of the developed methodologies to humans.

Invertebrate Model Daphnids were found to extensively metabolize testosterone to over 30 eliminated derivatives (4, 5). These elimination products consisted of hydroxylated derivatives, glucose conjugated derivatives, sulfate conjugated derivatives, and oxido-reduced products (Fig. 1). Glucose-conjugated testosterone was

the most abundant eliminated metabolite of testosterone by daphnids, constituting over 90% of the eliminated metabolites.

Fig. 1 Diagrammatic representation of the metabolism of testosterone by *Daphnia magna*. Intensity of the arrows depicts the relative contribution of each process to the metabolism of testosterone.

Methods are described in (4, 5).



Fish Model Fathead minnows eliminated testosterone using metabolic pathways similar to daphnids. Over twenty metabolites of testosterone were eliminated by the fathead minnow (6). Like daphnids, these products consisted of hydroxylated, oxidoreduced, and conjugated derivatives of testosterone. Testosterone was primarily conjugated to glucuronic acid in fathead minnows, not glucose as was observed in daphnids. Sex and age differences in the metabolic elimination of testosterone by fathead minnow were also characterized (6).

Mammalian Model Mice were found to biotransform testosterone in a manner qualitatively similar to the fathead minnow with the production of a variety of

hydroxylated, oxido-reduced, and conjugated derivatives. Significant sex differences in testosterone biotransformation were characterized in the mouse (e.g., Fig. 2). Hepatic testosterone hydroxylation profiles significantly differed between sexes. Further, males exhibited significantly higher testosterone glucuronosyl transferase activity and females exhibited significantly higher testosterone sulfotransferase activity (Fig. 2). These sex difference were found to be due primarily to differences in the regulation of these processes by testosterone (e.g. Fig 2).

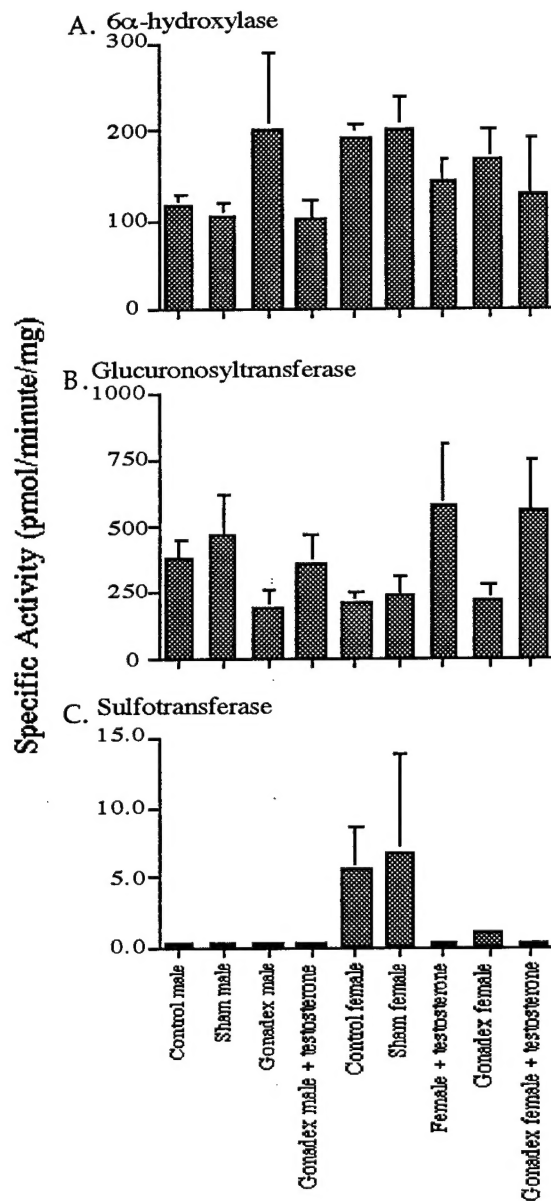
Results from this objective have conclusively demonstrated that testosterone is extensively biotransformed by qualitatively similar metabolic pathways in various organisms and that the metabolic elimination of testosterone can be measured using non-invasive approaches. The methodologies developed under this objective show promise for use in other invertebrate and vertebrate species including humans. In the course of this project, collaborative studies were undertaken that successfully applied these methodologies to the characterization of testosterone biotransformation by the mud snail (Dr. Eva Oberdorster, Tulane University), the blue crab (Dr. Dan Rittschof, Duke University), and the American alligator (Dr. Louis Guillette Jr., University of Florida).

Objective 2. Evaluate the utility of changes in the metabolic elimination of testosterone as a noninvasive biomarker of toxicant exposure.

Invertebrate Model Daphnids proved most useful in assessing the relationship between toxicant exposure and changes in the steroid hormone biotransformation due to their small size, availability, and amenability to laboratory experimentation. Daphnids were exposed to sublethal levels of 15 different chemicals and effects on the metabolic elimination of testosterone measured. Eighty percent of the chemicals

evaluated significantly altered at least one component involved in the metabolic elimination of testosterone.

Fig. 2 Hepatic testosterone 6 α -hydroxylase, UDP-glucuronosyl transferase, and sulfotransferase activities in male and female CD-1 mice and their modulation by gonadectomy and testosterone supplementation.



The most common effect was the inhibition of the glucose conjugation of testosterone (7). The elimination of hydroxylated derivatives of testosterone was also sensitive to chemical exposure with both increased (i.e. 4-nonylphenol (Fig. 3), tributyltin) and decreased (i.e. piperonylbutoxide, malathion) elimination of specific hydroxy metabolites observed. Some compounds (i.e. 4-nonylphenol (Fig. 3), piperonyl butoxide, pentachlorophenol, propiconazole) elicited a phenomenon that we refer to as *metabolic androgenization*. Metabolic androgenization is characterized by a decrease in the rate of production of glucose-conjugated testosterone with a commensurate increase in the rate of conversion of testosterone to variously androgenic derivatives (i.e. dihydrotestosterone, androstanediol) (8). We have shown that the androgenic derivatives of testosterone are preferentially retained by the daphnids (4). Thus the decrease in the major metabolic elimination process (glucose conjugation) with a commensurate increase in the rate of production of retained androgenic derivatives (oxido-reduced metabolites) would be predicted to elevate androgen levels within the organism. The causes and consequences of chemically-induced metabolic androgenization are discussed further under Objective 3.

Fish Model Experiments completed with the vertebrate models also demonstrated that chemical exposure can result in significant alterations in testosterone biotransformation. Exposure (7 days) of fathead minnow to concentrations of endosulfan approaching the chronic threshold concentration (i.e. chronic value) significantly reduced the metabolic elimination of several hydroxylated and reduced/dehydrogenated derivatives of testosterone but had no significant effect on the elimination of the conjugated derivatives of testosterone (Fig. 4).

A recently published study (9) has indicated that changes in steroid hormone metabolism following exposure to estrogenic chemicals is a more sensitive biomarker

of exposure as compared to vitellogenin induction in males, a more commonly used biomarker of exposure to environmental estrogens. Vitellogenin is a yolk precursor protein normally produced in response to estrogen stimulation in oviparous females. The detection of vitellogenin in the blood of males is indicative of exposure to an estrogen. We purified vitellogenin from the fathead minnow and produced polyclonal antibodies to this protein. The antibodies were used to develop a quantitative Enzyme-Linked Immuno Assay with which we can quantify vitellogenin levels in fathead minnow blood samples. This assay is currently being used to determine the relative sensitivities of changes in the metabolic elimination of testosterone and induction of vitellogenin as biomarkers of exposure to estrogenic chemicals. We are also using this assay as a means of exploring the relationship between changes in steroid hormone metabolism and vitellogenin induction/suppression in response to endocrine-disrupting chemicals.

Mammalian model Endosulfan was provided to mice in their diet for seven days at concentrations that approached the maximum tolerated oral dose. Endosulfan exposure significantly elevated the rate of production of several hydroxylated and reduced/dehydrogenated derivatives of testosterone in female mice and had no significant effect on testosterone conjugative biotransformations (Table 1 and Fig. 5). These inductive effects stand in contrast to the suppressive effects of endosulfan on testosterone biotransformation measured in the fish model.

Fig. 3 Metabolic elimination of [14 C]testosterone by daphnids following exposure to concentrations of 4-nonylphenol. Data are presented as the rate of elimination of: A. glucose-conjugated testosterone, B. sulfate-conjugated testosterone, C. oxido-reduced metabolites of testosterone, and D. hydroxylated metabolites of testosterone.

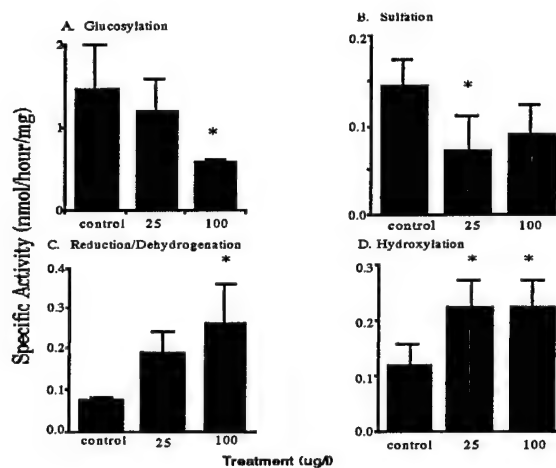


Fig. 4 Metabolic elimination of the major testosterone metabolites following seven days exposure of male fathead minnows to endosulfan

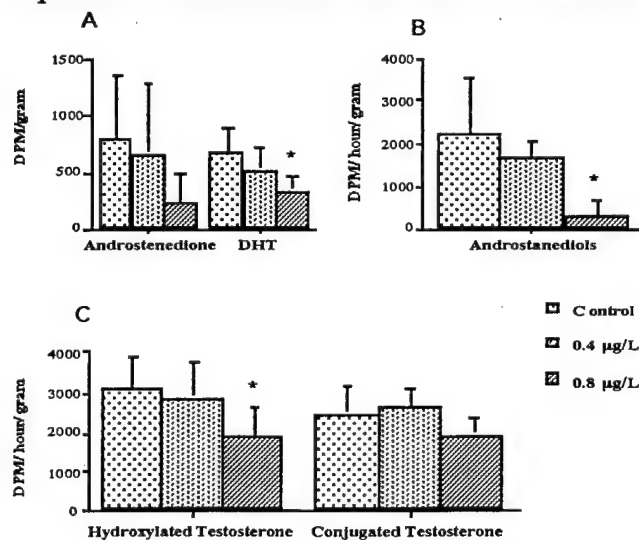


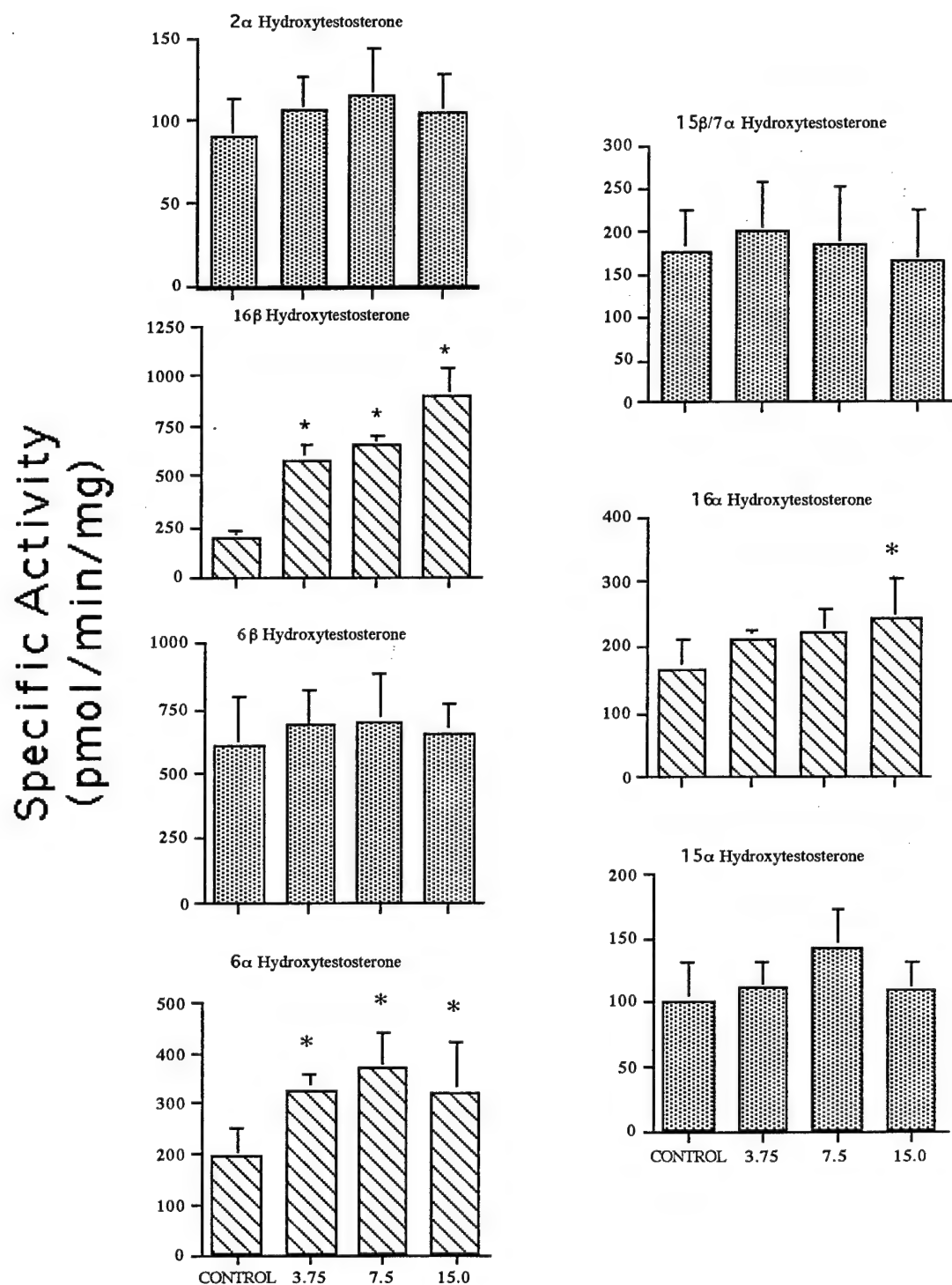
Table 1 Testosterone biotransformation rate for the major metabolite classes following seven days exposure of female mice to endosulfan.

Metabolite	Treatment, mg/kg ^a		
	Control	3.8	7.5
Total Hydroxy	1542 + 331	2220* + 257	2441* + 362
Androstenedione	657.3 ± 162.3	705.9 ± 57.7	842.1* ± 72.6
Glucuronic Acid Conjugate	69.7 + 14.7	73.52 ± 21.6	56.6 + 14.6
Sulfate Conjugate	2.1 + 1.2	1.8 ± 1.09	2.9 + 1.3

^aData are presented as mean ± standard deviation (pmol/min/mg).

*Significantly different from the control ($P \leq 0.05$).

Fig. 5 Testosterone hydroxylase activities in mice following seven days exposure to endosulfan (mg/kg).



Similar experiments performed with chlordecone, mirex, and phenobarbital also have demonstrated significant changes in testosterone biotransformation following toxicant exposure. *Results from this objective have definitively established that various components of the testosterone elimination pathway are susceptible to perturbation resulting from chemical exposure and, accordingly, can be exploited as a noninvasive biomarker of chemical exposure.* The final objective of the current program was to establish whether such perturbations are causally associated with disruptions in steroid hormone homeostasis and, as such, may serve as sensitive, predictive biomarkers of toxicant effects on steroid hormone-dependent processes such as development and reproduction.

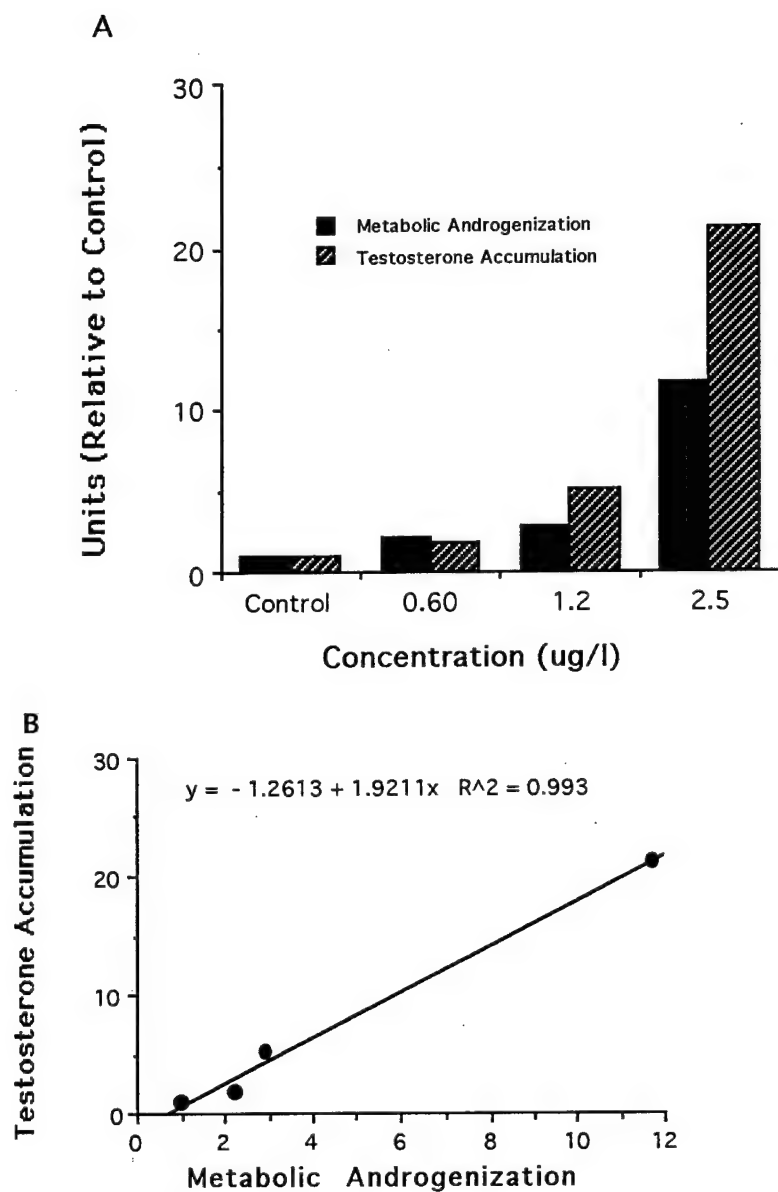
Objective 3. Establish relationships between chemical effects on the metabolic elimination of testosterone (the biomarker) and chronic toxicity.

Invertebrate Model The first step in evaluating whether chemical-induced perturbations in the metabolic elimination of testosterone are causally related to toxicity was to determine whether such perturbations alter testosterone homeostasis. Significant effort was expended towards the analyses of endogenous testosterone levels in daphnids. HPLC/immuno-radiometric procedures were developed whereby immunochemically-detectable testosterone could be measured in daphnids (~160 pg/mg soluble protein). However, antibody:antigen binding curves derived with daphnid samples had significantly different slopes than such curves derived with testosterone standards. These observations suggested that the immuno-detectable material in daphnids was not exclusively testosterone. Studies are underway utilizing GC/MS for the confirmed detection of endogenous testosterone in daphnids, as well as, the development of refined HPLC/immuno-radiometric techniques for the routine analyses of testosterone levels in this invertebrate.

Since endogenous testosterone levels could not be satisfactorily measured in daphnids, an alternative approach was utilized to provide insight into the effects of chemical exposure on testosterone homeostasis. We hypothesized that chemicals that cause metabolic androgenization as described above under *Objective 2* would cause an increase in the accumulation of exogenously-administered [^{14}C]testosterone. This hypothesis was tested by exposing daphnids to concentrations of the metabolically-androgenizing chemicals piperonylbutoxide, tributyltin, propiconazole, 4-nonylphenol and the nonandrogenizing chemicals endosulfan and clotrimazole. Daphnids were then exposed to [^{14}C]testosterone for 16 hours and the degree of accumulation of radioisotope measured. Chemicals that caused metabolic androgenization also increased the accumulation of [^{14}C]androgen by the daphnids and, for a given compound, the degree of metabolic androgenization and androgen accumulation were significantly correlated (e.g. Fig. 6). *These observations provide substantive evidence that chemically-induced metabolic androgenization alters testosterone homeostasis in daphnids.*

The observation that exposure to chemicals that cause metabolic androgenization results in increased accumulation of exogenous testosterone led us to develop a rapid screening test for chemically-induced metabolic androgenization (10). This assay involves exposing daphnids to concentrations of the chemical or environmental sample (i.e. waste-water sample) for 48 hours. [^{14}C]testosterone is then added to the medium for 16 hours during which time the daphnids accumulate the radiolabeled steroid hormone. Daphnids are then removed, rinsed, and radioactivity associated with the organisms is measured. Chemically-induced metabolic androgenization is indicated by the significant increase in radioactivity associated with the daphnids exposed to the chemicals as compared to controls.

Fig. 6 A. Effect of tributyltin on metabolic androgenization and [¹⁴C]testosterone accumulation by daphnids. B. Relationship between tributyltin-induced metabolic androgenization and accumulation of [¹⁴C]testosterone by daphnids.



Experiments were next conducted to establish whether metabolic androgenization was causally associated with toxicity. The following criteria were used to establish causality.

- 1) Toxicity consistently occurred with chemicals and at exposure concentrations that also caused metabolic androgenization.
- 2) Exposure to steroidal androgens mimicked the toxicity associated with chemically-induced metabolic androgenization.
- 3) Simultaneous exposure to a chemical that modulated toxicity of a steroidal androgen similarly modulated the toxicity a chemical that caused metabolic androgenization.

Daphnids were exposed to sublethal concentrations of various chemicals that were previously demonstrated to cause or not cause metabolic androgenization and effects on the steroid hormone-dependent processes associated with growth, development, and reproduction assessed. Treatments that caused $\geq 50\%$ change in the metabolic elimination of testosterone also reduced the number of offspring produced by parthenogenetically-reproducing daphnids and resulted in the release of under-developed offspring or eggs. Under-developed offspring were characterized as having poorly developed secondary antennae, an abnormally-shaped dorsal spine, and lipid deposits associated with the digestive tract. These daphnids typically died shortly following release from the female. *Thus, in accordance with criteria #1, chemicals that cause significant metabolic androgenization consistently elicited unique toxicity characterized by decreased fecundity and developmental abnormalities.*

Criteria #2 was evaluated by assessing the toxicity of the steroidal androgens testosterone and androstenedione over the life-cycle of the daphnids. Both compounds reduced fecundity and stimulated the release of underdeveloped offspring at concentrations significantly below exposure levels that were acutely toxic to the

daphnids. The steroidal androgens were more potent reproductive/developmental toxicants than any of the nonsteroidal compounds evaluated. Thus, *direct exposure to steroidal androgens mimicked the toxicity of chemicals that cause metabolic androgenization*. Criteria #2 for establishing causality was met.

Criteria #3 was evaluated using the chemical cyproterone acetate. Cyproterone acetate is an androgen antagonist with the mammalian androgen receptor. We hypothesized that cyproterone acetate would also interact with the putative daphnid androgen receptor, recognizing that this hypothesis was somewhat presumptive. Experiments were first conducted with cyproterone acetate alone to determine whether or not this compound elicited toxicity similar to a steroidal androgen in daphnids. Cyproterone acetate proved to be chronically toxic to daphnids; however, its toxicity did not resemble that of the steroidal androgens. Cyproterone acetone significantly reduced growth rates of exposed daphnids while not affecting molting rates (Table 2). Cyproterone acetate also reduced the number of offspring produced by female daphnids (Table 3). This effect appeared to be due to physical limitation as the small brood chambers of these organisms could not accommodate normal-sized clutches of egg. Most importantly, cyproterone acetate did not cause daphnids to release underdeveloped offspring. This developmental toxicity appears unique to steroidal androgens and chemicals that cause metabolic androgenization.

Co-exposure of daphnids to cyproterone acetate and testosterone had unexpected results. These compounds reduced the number of total offspring produced by daphnids in an additive manner (Table 3). However, cyproterone acetate potentiated the toxicity of testosterone with respect to the release of under-developed offspring (Table 4). Similar combination exposures were performed with cyproterone acetate and the metabolically-androgenizing chemical piperonyl butoxide. Again, the number

of offspring produced per daphnids was reduced from simultaneous exposure to these chemicals in an additive manner (Table 5). However, cyproterone acetate potentiated the developmental toxicity of piperonyl butoxide as measure by the release of under-developed offspring (Table 6). Thus consistent with criteria #3, *cyproterone acetate synergized with both a steroidal androgen (testosterone) and a chemical that causes metabolic androgenization (piperonyl butoxide)*. In summary, the three criteria selected to establish causality between chemically-induced metabolic androgenization and developmental toxicity were met.

Table 2. The effects of cyproterone acetate on growth and molting of daphnids during three-weeks exposure.

Concentration (μ M)	Length (mm)	Molts
0	4.48 \pm 0.10 ^a	8.7 \pm 0.5 ^a
0.31	..b	8.7 \pm 0.5
0.62	..b	9.1 \pm 0.3
1.2	4.23 \pm 0.13 ^c	9.1 \pm 0.6
2.5	3.73 \pm 0.11 ^c	9.4 \pm 0.7
5.0	2.72 \pm 0.22 ^c	8.3 \pm 0.5

^aMean \pm standard deviation

^bNot determined

^cSignificantly different from the control ($P \leq 0.05$)

Table 3 Effects of testosterone and cyproterone acetate alone and in combination on fecundity of daphnids

Testosterone (μ M)	Cyproterone Acetate (μ M)	Offspring/ Female	Percentage Reduction	
			Measured	Predicted ^b
0	0	156 \pm 15 ^a	--	--
0	1.2	139 \pm 16	11	--
0	2.5	110 \pm 9	29	--
0	5.0	27 \pm 8	83	--
6.2	0	123 \pm 17	21	--
6.2	1.2	90 \pm 16	42	32
6.2	2.5	57 \pm 15	63	50
6.2	5.0	12 \pm 5	92	100

^aMean \pm standard deviation

^bCalculated values are the sum of the percentage reduction in offspring production measured during exposure to 6.2 μ M testosterone with no cyproterone acetate and exposure to the indicated concentration of cyproterone acetate with no testosterone. Measured values were not significantly ($P \leq 0.05$) different from the respective predicted values. This indicates that the effects of the two compounds were additive.

Table 4 Effects of testosterone and cyproterone acetate alone and in combination on the release of under-developed neonates.

Testosterone (μ M)	Cyproterone Acetate (μ M)	Under-Developed Neonates (%)	
		Measured	Predicted ^b
0	0	0 \pm 0 ^a	--
0	1.2	0 \pm 0	--
0	2.5	0 \pm 0	--
0	5.0	0 \pm 0	--
6.2	0	20 \pm 9	--
6.2	1.2	60 \pm 19	20
6.2	2.5	88 \pm 9	20
6.2	5.0	87 \pm 17	20

^aMean \pm standard deviation

^bPredicted values are the sum of the percentage under-developed neonates measured during exposure to 6.2 μ M testosterone with no cyproterone acetate and during exposure to the indicated concentration of cyproterone acetate with no testosterone. Measured values significantly differed ($P=0.024$) from the respective predicted values indicating that the combined effects of cyproterone acetate and testosterone were greater than additive.

Table 5 Effects of piperonyl butoxide and cyproterone acetate alone and in combination on fecundity of daphnids

Piperonyl Butoxide (μ M)	Cyproterone Acetate (μ M)	Offspring/ Female	Percentage Reduction	
			Measured	Predicted ^b
0	0	139 \pm 26 ^a	--	--
0	1.2	142 \pm 16	0	--
0	2.5	105 \pm 15	24	--
0	5.0	16 \pm 7	88	--
1.0	0	78 \pm 25	44	--
1.0	1.2	36 \pm 13	74	44
1.0	2.5	31 \pm 9	78	68
1.0	5.0	1 \pm 1	99	100

^aMean \pm standard deviation

^bCalculated values are the sum of the percentage reduction in offspring production measured during exposure to 1.0 μ M piperonyl butoxide with no cyproterone acetate and exposure to the indicated concentration of cyproterone acetate with no piperonyl butoxide. Measured values were not significantly ($P \leq 0.05$) different from the respective predicted values. This indicates that the effects of the two compounds were additive.

Table 6 Effects of piperonyl butoxide and cyproterone acetate alone and in combination on the release of under-developed neonates.

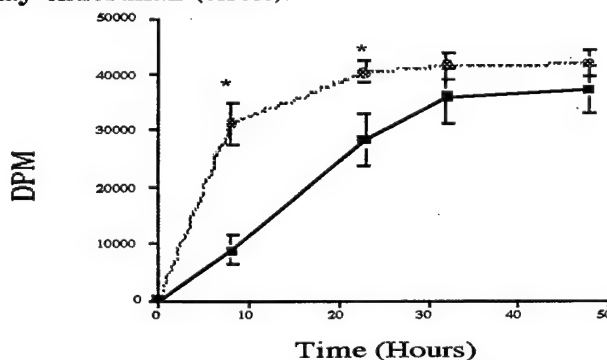
Piperonyl Butoxide (μ M)	Cyproterone Acetate (μ M)	Under-Developed Neonates (%)	
		Measured	Predicted ^b
0	0	7 \pm 5 ^a	--
0	1.2	8 \pm 10	--
0	2.5	2 \pm 4	--
0	5.0	0 \pm 0	--
1.0	0	36 \pm 19	--
1.0	1.2	87 \pm 26	44
1.0	2.5	80 \pm 16	38
1.0	5.0	100 \pm 0	36

^aMean \pm standard deviation

^bCalculated values are the sum of the percentage under-developed neonates measured during exposure to 1.0 μ M piperonyl butoxide with no cyproterone acetate and during exposure to the indicated concentration of cyproterone acetate with no piperonyl butoxide. Measured values significantly differed ($P=0.020$) from the respective predicted values indicating that the combined effects of cyproterone acetate and testosterone were greater than additive.

Vertebrate Models Experiments conducted with the vertebrate models also demonstrated that perturbations in the metabolic elimination of testosterone can alter steroid hormone homeostasis. For example, endosulfan treatment not only elevated the rate of production of several testosterone hydroxyl metabolites in mice (Fig. 5) but also significantly increased the rate of urinary elimination of testosterone (Fig. 7) (11). Nonetheless, these significant effects on testosterone metabolism and clearance resulted in only a small and statistically insignificant effect on serum testosterone levels (11). The attenuated effect on serum testosterone levels was likely due to homeostatic mechanisms (hypothalamic:pituitary feedback mechanisms (12)) that caused an increase in testosterone synthesis. Mice used in this study were not synchronized with respect to the estrus cycle. Synchronization, as will be established in future experiments, will reduce the variability associated with analyses of serum hormone levels, perhaps allowing for the detection of significant differences between chemical treated and untreated animals.

Fig. 7. Urinary elimination of [^{14}C]testosterone by control mice (square) and following exposure to 7.5 mg/kg/day endosulfan (circle).



Results of mouse exposure to 4-nonylphenol have indicated that homeostatic mechanisms can also contribute to perturbations in steroid homeostasis (13). 4-Nonylphenol-exposed mice experienced a significant enlargement of the prostate

gland and this increase was associated with an increase in serum testosterone levels. The increase in serum testosterone was not due to alterations in testosterone biotransformation and elimination, but rather was due to an increase in the rate of gonadal synthesis of testosterone. Experimentation continues to definitively establish the mechanism by which 4-nonylphenol alters androgen homeostasis resulting in enlarged prostate. Data thus far suggest that 4-nonylphenol is functioning as an anti-androgen at the levels of the hypothalamus and/or pituitary gland preventing the feedback suppression of gonadal testosterone synthesis. As a result, testosterone synthesis is stimulated, serum testosterone levels increased causing an increase in prostate gland mass. Currently unresolved is why anti-androgenic activity of 4-nonylphenol is not also elicited with the prostatic androgen receptors which would then result in a decrease in prostate size. Tissue-specific differences in 4-nonylphenol availability, metabolism, or receptor type may be responsible for this apparent incongruity.

Taken together, results from this research program have amply demonstrated that changes in the metabolic elimination of testosterone can serve as a sensitive, predictive, noninvasive biomarker of toxicity associated with hormonal perturbation. These results have direct value in providing approaches for the toxicity characterization of chemicals or the detection of environmental contaminants. Indeed, this type of approach has been identified by the Environmental Protection Agency (14) and the United Kingdom's Institute for Environment and Health (15) as an area that should be explored further for the identification and characterization of environmental endocrine disruption. Further, the Principal Investigator has been invited to present a plenary session address on these methodologies at the 1998 American Society of Testing and Materials meeting entitled *Standardization of Biomarkers for Endocrine Disruption and Environmental Assessment*. The Principal

Investigator has received financial support from the U.S. Environmental Protection Agency to continue the invertebrate studies initiated in this program with the ultimate goal of establishing measurable endpoints of environmental endocrine disruption in these organisms.

Experimental results from this program also provide a solid scientific foundation upon which to hypothesize that alterations in the metabolic elimination of steroid hormones by humans may serve as a means of diagnosing exposure to and toxicity of environmental contaminants. A grant proposal has been submitted to the U.S. Air Force, Office of Scientific Research for the development of diagnostic procedures for the detection of endocrine disruption in humans.

Methods Developed and Used During this Research Program

Method #1 Serum estradiol and testosterone measurements

Blood samples were allowed to clot at room temperature and serum was obtained by centrifugation (16). Serum from each sample was immediately frozen at -20°C until assayed. Total testosterone and 17β -estradiol in serum was measured within 5 days of preparation by solid-phase radioimmunoassay using commercially available reagents and protocols (Diagnostic Products Corp., Los Angeles, CA).

Method #2 Microsome and cytosol preparation

Individual mouse or fish liver samples were thawed and homogenized on ice in chilled cytosol buffer (0.1 M Hepes (pH 7.4), 1 mM EDTA, 10% glycerol) with a Dounce homogenizer. Microsomes were prepared by differential centrifugation (17). Cytosolic supernatant were reserved, microsomal pellets will be resuspended in microsomal buffer (0.1 M potassium phosphate (pH 7.4), 0.1 mM EDTA, 20% glycerol), and both tissue preparations were stored at -80°C until used in enzyme assays. Protein concentrations of the tissue preparations were determined (18) using commercially prepared reagent (Biorad) and bovine serum albumin (Sigma, St. Louis, MO) as a standard.

Method #3 Testosterone hydroxylase and oxido-reductase activities

Testosterone hydroxylase and oxido-reductase activities were assayed as previously described (19, 20) using 400 µg microsomal protein and 40 nmol [¹⁴C]testosterone as substrate (1.8 mCi/mmol, Dupont NEN, Boston, MA) in 0.1 potassium phosphate buffer (pH 7.4). Reactions were conducted at 37°C and initiated with 1 mM NADPH. Total assay volume was 400 µl. The reactions were terminated after 10 min. by addition of 1 ml ethyl acetate. Each tube was vortexed approximately 30 seconds and then centrifuged for 5 min. to separate the ethyl acetate and aqueous phases. Ethyl acetate fractions were transferred to a separate tube. The aqueous phase was extracted with ethyl acetate a total of 3 times (1,2,1 ml) to ensure recovery of all hydroxy metabolites. Ethyl acetate fractions derived from extraction of the same aqueous sample were combined and then evaporated under a stream of nitrogen to dryness. The residue was resuspended in 70 µl ethyl acetate (35 µl X 2), and metabolites separated by thin layer chromatography. Unmetabolized [¹⁴C]testosterone and individual [¹⁴C]testosterone metabolites were identified as we have previously described and quantified by liquid scintillation

spectroscopy (20). Specific activity for each metabolite was calculated as pmol of metabolite produced/min/mg of microsomal protein.

Method #4 UDP-Glucuronosyltransferase activity

UDP-glucuronosyltransferase activity towards [^{14}C]testosterone was assayed under conditions previously described (21) with the following modifications. Microsomal protein (200 μg) were incubated at 37°C with 40 nmol [^{14}C]testosterone (1.8 $\mu\text{Ci}/\mu\text{mol}$) in 0.1 M potassium phosphate buffer, pH 7.4. Reactions were initiated with 10 μL uridine 5'-diphosphoglucuronic acid in buffer (12.9 mg/ml, Sigma) to provide a total assay volume of 400 μL and terminated after 10 minutes by addition of 2 ml ethyl acetate and vortexing. Product formation was shown to be linear over this time period. Phases were separated by centrifugation and ethyl acetate fractions removed. The extraction procedure was repeated a second time with an additional 2 ml of ethyl acetate. Glucuronide conjugates were quantified by liquid scintillation counting of a 100 μL aliquot from the post-extraction aqueous phase. Samples were run with every assay that consist of all constituents except microsomes. These samples were used to correct for any spontaneous conjugation of glucuronic acid to the testosterone and any [^{14}C]testosterone that was not extracted from the aqueous phase by the ethyl acetate. Radioactivity in these aqueous samples, following ethyl acetate extraction (typically 0.1% of the total radioactivity in the assay), were subtracted from the total radioactivity associated with the glucuronic acid-conjugated testosterone in each assay. Specific activity was calculated as pmol of conjugate produced/min/mg microsomal protein.

Method #5 Sulfotransferase activity

Cytosolic protein (200 μg) was incubated with 40 nmol [^{14}C]testosterone (1.8 $\mu\text{Ci}/\mu\text{mol}$) in 0.1 M potassium phosphate buffer (pH 6.5) to assay for activity of sulfotransferase enzymes

toward testosterone. Reactions were initiated with 10 μ l adenosine 5'-phosphosulfate (10.1 mg/ml, Sigma) for a total assay volume of 400 μ l. Assay tubes were covered, and incubated in 37°C water bath for 20 hours. Product formation was shown to be linear over this time period. Reactions were stopped by addition of 2 ml ethyl acetate and vortexing. Unconjugated testosterone was removed by ethyl acetate extraction (2X, 2 mL each). Sulfate conjugates were quantified by scintillation counting of a 100 μ L aliquot from the post-extraction aqueous phase. Approximately 70% of the sulfate conjugates were retained in the aqueous phase following organic extraction; therefore, sulfate conjugates extracted into the ethyl acetate were separated from the [14 C]testosterone by TLC using an 80% methylene chloride:20% acetone solvent system. Sulfate-conjugated [14 C]testosterone were then be cut from the TLC plate and quantified by liquid scintillation counting. Total dpms associated with the sulfate conjugated [14 C]testosterone (aqueous and organic extracted) were combined to calculate specific activity (pmol/min/mg)

Method #6 [14 C]Testosterone elimination by mice

Following treatment with the chemical under study, mice were be injected (i.p.) with 125,000 dpm [14 C] testosterone (57 μ Ci/ μ mol) in 100 μ l corn oil. Mice were housed individually, in metabolism cages, with water provided *ad libitum*. Urine and feces were collected from each cage at 8, 24, 32, and 48 hours after injection. Preliminary experiments had shown that >90% of the administered radioactivity is eliminated during the first 48 hours. Urine and feces from each animal at each time point was stored in separate, sealed containers at -20°C until the study was completed. [14 C]testosterone elimination in urine was quantified by scintillation counting of a 100 μ l aliquot of urine from each animal at each time point.

Feces was dried in a 100°C oven for 16 hours, weighed, and ground with a mortar and pestle to produce a homogeneous mixture. Two samples of ground feces (50 mg each) from each animal at

each time point was oxidized (R.J Harvey Instrument Corp., Hillsdale NJ). [^{14}C]carbon released from each sample will be trapped into vials of scintillation cocktail (Harvey Instrument Corp.) and quantified by liquid scintillation spectroscopy.

Method # 7 [^{14}C]Testosterone metabolite elimination by aquatic species Testosterone metabolism in daphnids and fish was assessed by incubating the organisms with [^{14}C]testosterone (2 mCi/mmol, 150,000 dpm, Dupont, Boston, MA)) for various time periods. Following exposure, testosterone, its hydroxyl, reduced and dehydrogenated metabolites were extracted from the aqueous medium with ethyl acetate. Metabolites were separated by thin layer chromatography using a solvent system of methylene chloride:acetone (4:1 v,v) followed by chloroform:ethylacetate:ethanol (4:1:0.7, v/v/v) and individual metabolites were quantified by scintillation spectroscopy. Post extraction aqueous samples contained glucuronic acid, glucose, and sulfate conjugated testosterone metabolites. These samples were sequentially incubated with glucuronidase, glucosidase or sulfatase enzymes (Sigma) to hydrolyze the conjugates. Deconjugated testosterone and deconjugated testosterone metabolites were extracted and analyzed as described for the initial ethyl acetate extracts.

Method # 8 Rapid Analyses of Metabolic Androgenization Adult daphnids were exposed to concentrations of the chemical under evaluation for metabolic androgenization for 64 hours. [^{14}C]testosterone was added to the assay mixtures for the final 16 hours and the daphnids allowed to accumulate radioisotope. Daphnids were then harvested, rinsed, and radioactivity associated with the organisms was measured by scintillation spectroscopy. Exposure to chemicals that cause metabolic androgenization resulted in significant increases in the accumulation of [^{14}C]androgen by the daphnids as compared to unexposed controls.

Method #9 Vitellogenin Purification Plasma proteins from 17 β -estradiol-induced male fathead minnows were separated on a DEAE-agarose column using a salt gradient.

The elution profile of the proteins was monitored at 280 nm and individual proteins collected. Isolated vitellogenin was identified based upon: a) molecular weight, b) immunoreactivity with antibodies to vitellogenin from other species, and c) amino acid composition.

Method #10 Vitellogenin Antibody Production Adult male fathead minnows were injected with 17β -estradiol to induce the production of vitellogenin. Induction was confirmed by SDS-polyacrylamide gel electrophoresis with proteins stained with coomassie brilliant blue and vitellogenin identified by comparison to purified striped bass vitellogenin. Vitellogenin-containing plasma (300 μ l) was mixed with 700 μ l of phosphate-buffered saline and the mixed 1:1 with Freud's complete adjuvant. This mixture was injected subcutaneously into two female rabbits at four sites along each rabbit's back (1 ml per rabbit). This procedure was repeated six times but with incomplete Fred's adjuvant over 3.5 months. Blood samples were periodically collected and, when the antibody titer was sufficiently high, the rabbits were euthanized and blood collected. Antibody-containing serum was prepared from the blood and mixed 1:1 with plasma from males that were not treated with 17β -estradiol to bind and inactivate antibodies present in the serum that recognize male plasma proteins.

Method #11 Vitellogenin Quantification: Competitive ELISA Methods to quantify vitellogenin levels in serum collected from fathead minnows was based upon those described by Specker and Anderson (22) and summarized below.

- 1) Microtiter plate wells were coated with purified fathead minnow vitellogenin.
- 2) Serial dilutions of the vitellogenin were prepared and incubated with a fixed amount of vitellogenin antibody (standard curve).
- 3) Plasma samples were incubated with the same concentration of antibody used in step 2.

- 4) Antibody-standard curve solutions and antibody-plasma solutions were added to the microtiter plate wells that were coated with vitellogenin in step 1.
- 5) Solutions were incubated for 1 hour at 37° C.
- 6) Unbound materials was rinsed from the wells
- 7) Horseradish peroxidase-linked secondary antibody was added to the wells and incubated for 1 hour at 37° C.
- 8) Wells were rinsed and horseradish peroxidase chromogenic substrate was added to the wells.
- 9) Color generation was stopped after sufficient time with 6 N HCl.
- 10) Absorbance associated with each well was measured with a microtiter plate reader.
- 11) Absorbance values are used to calculate the concentration of vitellogenin associated with the plasma samples from the standard curve generated with purified vitellogenin.

Personnel

<u>Name</u>	<u>Dates of involvement</u>	<u>Role</u>
Gerald A. LeBlanc	1994-present	Principal Investigator
Damian Shea	1994-present	Co-Investigator (Analytical Chemistry)
William Baldwin	1994	Grad. Student: steroid metabolism in daphnids
Jim Yates	1994	Grad. Student: steroid metabolite analyses
Louise Parks	1994-present	Grad. Student: steroid metabolism in daphnids and fish
Lucy Lopez	1995-1996	Technician: steroid metabolism in daphnids and fish
Charles Olsen	1995-1997	Technician (part time): culture maintenance
James McLachlan	1995-present	Technician: steroid metabolism in daphnids and mice

Catherine St. Amour	1996	Grad. Student: steroid metabolism in daphnids
Vickie Wilson (no stipend)	1996-present	Grad. Student: steroid metabolism in mice
Lisa Bain	1996	Grad Student: steroid metabolism in mice

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Appendix I. INTERACTIONS/TRANSITIONS: Publications and presentations resulting from this research program

- Baldwin W.S., Milam D.L., and LeBlanc G.A. 1995. Physiological and biochemical perturbations in *Daphnia magna* following exposure to the model environmental estrogen diethylstilbesterol. *Environ. Toxicol. Chem.* 14:945-952.
- LeBlanc G.A. 1995. Are environmental sentinels signaling? *Environ. Health Perspect.* 10:888-890.
- Parks L.G. and LeBlanc G.A. 1996. Reductions in steroid hormone biotransformation/elimination as a biomarker of pentachlorophenol chronic toxicity. *Aquatic Toxicol.* 34(4):291-303.
- LeBlanc G.A. 1997. Invertebrates as sentinels of xenobiotic-induced endocrine disruption. in: *Endocrine Disruptors: Advances in Measuring and Analyzing Their Effects*. (P. Guttry ed.) IBC Library Series, Southborough, MA. 2:1-24.
- LeBlanc G.A., Bain L.J., and Wilson V.S. 1997. Pesticides: multiple mechanisms of demasculinization. *Mol. Cell. Endocrin.* 126:1-5.
- LeBlanc G.A. and Bain L.J. 1997. Chronic toxicity of environmental contaminants: sentinels and biomarkers. *Environ. Health Perspect.* 105(Suppl. 1):65-80.

Baldwin W.S., Graham S.E., Shea D., and LeBlanc G.A. 1997. Metabolic androgenization of female *Daphnia magna* by the xenoestrogen 4-nonylphenol, *Environ. Toxicol. Chem.* 16:1905-1911.

LeBlanc G.A. 1997. Chemical Contamination Affects Wildlife In: *Current Controversies: Garbage & Waste*, Greenhaven Press, Inc. San Diego. 92-95.

In press or submitted

LeBlanc G.A. 1997. Steroid hormone-regulated processes in invertebrates and their susceptibility to environmental endocrine disruption. In press, *Environmental Endocrine Disruptors: An Evolutionary Perspective*, Taylor and Francis Publishers.

Oberdorster E. and LeBlanc G.A. 1997. Alterations in metabolism of [¹⁴C]testosterone following exposure of *Daphnia magna* to tributyltin, In press, *Arch. Environ. Contam. Toxicol.*

Baldwin W.S., Graham S.E., Shea D., and LeBlanc G.A. Altered metabolic elimination of testosterone and associated toxicity following exposure of *Daphnia magna* to nonylphenol polyethoxylate. In press, *Ecotoxicol. Environ. Safety*.

Wilson V.S. and LeBlanc G.A. Endosulfan elevates testosterone metabolism and clearance in CD-1 mice. In press, *Toxicol. Appl. Pharm.*

In preparation

Parks L.G. and LeBlanc G.A. Characterization of testosterone metabolic elimination pathways in the fathead minnow (*Pimephales promelas*): Utility as a biomarker of endocrine disruption.

Parks L.G. and LeBlanc G.A. Altered metabolic elimination of testosterone by fathead minnows (*Pimephales promelas*) following exposure to endosulfan.

Parks L.G., LeBlanc G.A., and Sullivan C. Development of antibodies to fathead minnow (*Pimephales promelas*) vitellogenin for use in the detection of exposure to xenoestrogens.

Wilson, V.S. and LeBlanc G.A. Sex differences in the hepatic biotransformation of testosterone by CD-1 mice: Biomarkers of endocrine disruption.

Wilson V.S., McLachlan J.B., and LeBlanc G.A. Mirex and chlordecone induce steroid hydroxylase activity and enhance the urinary elimination of testosterone.

LeBlanc G.A. and McLachlan J.B. Altered testosterone homeostasis and prostatic hyperplasia in mice exposed to 4-nonylphenol.

LeBlanc G.A. Chemically-induced metabolic androgenization disrupts embryo development and reduces fecundity of daphnids (*Daphnia magna*).

LeBlanc G.A. Endpoints for the rapid detection of chemically-induced endocrine disruption in invertebrates.

LeBlanc G.A. and McLachlan J.B. Alterations in the metabolic elimination of testosterone by *Daphnia magna* following acute exposure to tributyltin.

LeBlanc G.A. and McLachlan J.B. Temporal decline in the male:female birth ratio: Is chemical-induced hormone disruption the cause?

Presentations

LeBlanc G.A., Milam D.L. and Baldwin W.S. 1994. Developmental and reproductive effects of the model environmental estrogen diethylstilbesterol (DES) in *Daphnia magna*. Society of Environmental Toxicology and Chemistry, 15th Annual Meeting, Denver CO.

Parks L.G. and LeBlanc G.A. 1995. Inhibition of steroid glucosyl transferase activity by pentachlorophenol at concentrations that reduce survival and fecundity of *Daphnia magna*. Society of Toxicology , 34th Annual Meeting, Baltimore MD.

Oberdorster E., LeBlanc G.A., and Rittschof D. 1995. Acute and chronic effects of Tributyltin on *Daphnia magna*: Alterations of testosterone metabolism. 5th COMTOX Symposium on Toxicology and Clinical Chemistry of Metals, Vancouver, BC, Canada.

LeBlanc G.A., Baldwin W.S., Parks L.G., and Oberdorster E. 1995. Relationship between alterations in steroid hormone metabolism and chronic toxicity of endocrine-disrupting chemicals. 7th Meeting of the International Congress of Toxicology, Seattle WA.

LeBlanc G.A., Parks L.G., Baldwin W.S., McLachlan J.B., and Oberdorster E. 1995. Altered steroid hormone metabolic profiles as a biomarker of reproductive toxicity of endocrine-disrupting chemicals. 2nd SETAC World Congress, Vancouver, BC, Canada.

Parks L.G. and LeBlanc G.A. 1995. Pentachlorophenol disrupts steroid hormone metabolism at concentrations that reduce survival and fecundity of *Daphnia magna*. Proc. 2nd SETAC World Congress, Vancouver, BC, Canada.

LeBlanc G.A., Lopez L., and McLachlan J.B. 1996. Comparative study of the endocrine-disrupting toxicity of the fungicides propiconazole and clotrimazole. Society of Environmental Toxicology and Chemistry, 17th Annual Meeting, Washington DC.

Baldwin W.S., Graham S.E., Shea D., and LeBlanc G.A. 1996. Metabolic androgenization of female *Daphnia magna* by the xenoestrogen 4-nonylphenol. Society of Environmental Toxicology and Chemistry, 17th Annual Meeting, Washington DC.

Parks L.G. and LeBlanc G.A. 1996. Characterization of testosterone metabolic clearance pathways in fathead minnow for use in assessing endocrine

disruption. Society of Environmental Toxicology and Chemistry, 17th Annual Meeting, Washington DC.

Parks L.G., Heppel S.A., Shea D., Sullivan C.V., and LeBlanc G.A. 1997.

Vitellogenin detection in two cyprinid species using a competitive ELISA in laboratory and field studies. Society of Environmental Toxicology and Chemistry, 18th Annual Meeting, San Francisco, CA.

Invited Presentations (G.A. LeBlanc)

Environmental Endocrine Disrupters, expert panel discussion. 1994. Society of Environmental Toxicology and Chemistry, Carolinas Chapter, Duke University, Durham, North Carolina.

Endocrine-Disrupting Chemicals: Mechanisms of Action and Ecotoxicological Implications. 1995. Duke University, Durham, North Carolina.

Ecotoxicological Consequences Endocrine Disruption in Invertebrates. 1995. U.S. EPA-sponsored workshop entitled "Endocrine-Disrupting Chemicals in the Environment: Research Needs, Duluth, Minnesota.

Perturbations in Steroid Hormone Metabolism by Environmental Endocrine Disrupters, Common Responses from Arthropods to Humans. 1995. Maine Toxicology Institute, University of Maine, Orono, ME.

Altered Steroid Hormone Metabolic Profiles as a Biomarker of Reproductive Toxicity of Endocrine-Disrupting Chemicals. 1995. Duke University Marine Laboratory, Beaufort, North Carolina.

Invertebrates as Sentinels of Xenobiotic-Induced Endocrine Disruption. 1996. Endocrine Disrupters: Advances in Measuring and Analyzing Their Effects, Washington, DC.

Steroid Hormone Metabolism: Targets and Biomarkers of Endocrine-Disrupting Chemicals. 1996. Duke University Spring Toxicology Symposium, Duke University, Durham, NC.

Mechanisms by Which Chemicals Alter Steroid Homeostasis. 1996. North Carolina State University, Raleigh, NC.

Steroid Metabolism as a Biomarker of Toxicant Exposure and Effect. 1996. Wright Patterson Air Force Base, Fairborn, Ohio.

Chemically-Induced Metabolic Androgenization: A Mechanism of Endocrine Disruption Common to Both Invertebrates and Vertebrates. 1996. Georgia Institute of Technology, Atlanta, Georgia.

Susceptibility of Invertebrates to Sex Hormone Disruption. 1997. Workshop on the Effects of Sex Hormone Disrupters in Wildlife and Ecosystems. University of Leicester, Leicester, UK.

Aquatic Invertebrates: Models for the Study of Environmental Endocrine Disruption.

1997. Nicholas School of the Environment, Duke University, Durham, NC.

Research Needs and Testing Strategies for the Detection of Environmental Endocrine Disruptors. 1997. Second EPA/CMA/WWF Workshop on Environmental Endocrine Disruption, Kansas City, Missouri.

Approaches for the Rapid Assessment of Endocrine-Disrupting Toxicity in Invertebrates. 1998 (scheduled). American Society for Testing and Materials, Atlanta, Georgia.

New discoveries, inventions, or patent disclosures

Many of the assays and biomarkers developed under this program are currently under evaluation by United States and European regulatory agencies as screening methods for evaluating the endocrine-disrupting properties of chemicals. Metabolic androgenization in daphnids and vitellogenin induction in fathead minnows has received the greatest attention and will likely be adopted as standard methods for the evaluation of endocrine disrupting chemicals. We are currently exploring patent opportunities with respect to these two assays.